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(56) Documents Cited
GB 2166545 A GB 2133007 A EP 0596355 A1
EP 0037583 A1 WO 96/13607 A1 WO 91/16336 A1
WO 80/02295 A1 WO 80/00351 A1 US 5399487 A
US 5236827 A US 5184300 A

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(54) Monitoring an enzyme involving a substrate therefor labelled with a fluorophore

(57) A method of monitoring the presence of an enzyme comprises exposing a substrate, labelled with a fluorophore, to the enzyme, and monitoring the fluorescently-labelled products thus obtained, preferably with a fluorometer. The enzyme may be a protease (preferably subtilisin, esperase, trypsin or alcalase), cellulase, lipase, collagenase or amylase. The substrate for protease may be a protein (preferably gelatin, porcine thyroglobulin or bovine serum albumin) and that for cellulase may be cellulose. Preferred fluorophores are lucifer yellow and fluorescein. The method may be performed by conveying air containing the enzyme into a liquid, and passing the resulting enzyme solution over a fixed bed of the labelled substrate, which may be on a support, preferably selected from glass beads or cellulose particles containing iron oxide.

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Method for Monitoring Enzymes

The present invention is concerned with monitoring for the presence and/or determining the concentration of enzymes and in particular with a method which makes possible such monitoring on a continuous basis.

Enzymes of various types are commonly used both in scientific research and in manufacturing industry, in particular the detergent and food industries. However it is important to monitor the exposure of workers to enzymes because some enzymes can have deleterious health effects, for example leading to respiratory sensitisation. Ideally monitoring for enzymes should be carried out either at regular intervals to ensure that exposure limits are not exceeded or more preferably continuously to detect concentrations above such limits and thereby make possible immediate corrective action.

Available methods for monitoring enzymes involve sequences of operations which typically include collecting an air sample, extracting solid material from the air, eluting the enzymes into solution, initiating the enzyme reaction, producing a derivative of the reaction product and measuring the quantity of the derivative by, for example, a spectrophotometric method. Such sequences of steps are inevitably time-consuming, are necessarily carried out in a laboratory away from the monitoring site, and therefore are generally unsuitable for continuous or frequent on-site monitoring.

It is an object of the present invention to provide an improved method of monitoring enzymes, which is suitable for use on a continuous basis or for monitoring at frequent short intervals.

The method according to the present invention for monitoring for the presence of an enzyme comprises producing a substrate for the enzyme, which substrate has been modified by labelling it with a fluorophore, exposing the labelled substrate to the enzyme, and monitoring the fluorescent-labelled reaction products thereby produced. The fluorescence response, for example as determined by means of a fluorimeter, has proved to be a reliable and effective indication

of the presence and, subject to calibration, the concentration of the enzyme detected.

Enzymes, to the monitoring of which the method of the present invention may be applied, include enzymes of the protease, cellulase, lipase, collagenase and amylase types, for example subtilisin, esperase, trypsin and alcalase.

The substrate used in the method is selected from among those with which the enzyme to be monitored reacts. Thus, by way of example, for monitoring of the protease enzymes subtilisin, esperase, trypsin and alcalase, the substrate selected is preferably a protein. Proteins which have been used successfully in experiments for this purpose include gelatin, porcine thyroglobulin (PTG) and bovine serum albumin (BSA). Cellulases have been successfully monitored using labelled cellulose and collagenases have been successfully monitored with labelled collagen as the substrate.

The fluorophore used in the method of the invention to label the substrate is selected to be readily and consistently detectable by normal fluorometric methods. Preferably the selected fluorophore is not influenced by variations in ambient conditions such as pH level. The preferred

fluorophore is lucifer yellow. Less preferred, since the fluorescence signal arising is markedly influenced by PH levels, are fluorescein and its derivatives, for example fluorescein isothiocyanate (FITC).

The method can be put into practice in various ways but it is much preferred to practise it on a continuous basis by conveying the air which contains the enzyme continuously into a collector containing a solution which is then passed over a fixed bed of the labelled substrate in a reactor, in particular a small reaction vessel which may be carried to, or installed at, the place to be monitored. The labelled substrate is in many cases advantageously carried upon a suitable support, for example glass beads or magnetisable particles of cellulose containing iron oxide. In the case of cellulose however, in view of its insolubility in water, the substrate may be used as solid particles without such support.

In one alternative way of practising the method according to the present invention, the labelled substrate is supported upon an optical fibre and the fluorescence signal along the interior of the fibre is monitored continuously. The signal will initially have a relatively higher value

and, as the labelled reaction products break away from the fibre in response to the presence of enzyme, the signal will drop accordingly.

The reproducibility and sensitivity of the results obtained by the method of the invention is improved by choosing a solution of a buffer of the correct pH for passing through the reactor. For example, in the case of cellulose-based reactions, the use of acetate buffer is to be recommended, to maintain the pH value at a level corresponding to maximal enzyme activity. When the substrate is supported upon glass beads, the preferred buffer for most proteases, giving a pH of 7.4, is PBST (phosphate-buffered saline with Tween 20). The reproducibility of the fluorescence signals is further enhanced by the inclusion of a small quantity of a detergent in the liquid.

The method according to the invention is illustrated, by way of example only, by means of the following Examples.

Example 1.

Gelatin labelled with the fluorophore lucifer yellow and supported upon cellulose particles was loaded into a mini-bioreactor and samples comprising 10ng of the enzyme trypsin, buffered with tris-(hydroxymethyl)-aminomethane and containing 0.1 per cent of the detergent

Tween 20, were fed through the reactor at flow rates of 0.8 ml/min and 1.5 ml/min respectively. Within response times of the order of 1 minute, fluorescence responses of the order of 45 and 35 fluorescence units respectively were obtained.

Example 2.

Bovine serum albumin labelled with the fluorophore fluorescein and supported upon cellulose particles were packed into a mini-column and this bioreactor was fed, at a flow rate of 1.0 ml/min, with samples containing 0.25 to 5 ng of the enzyme subtilisin, buffered with PBST (pH 7.4), which also contains 0.1% (v/v) Tween 20 (sorbitan monolaurate polyoxyalkylene). Within a response time of 60 seconds, a fluorescence peak was observed, the height of which was found to correspond to the amount of enzyme fed.

Example 3.

Cellulose particles activated and then labelled with the fluorophore lucifer yellow were packed into a mini-column and this bioreactor was fed, at a flow rate of 2 ml/min, with samples containing 0.5 to 10 units per millilitre of cellulase activity, buffered with 0.05 mol/l acetate (pH 5.0), which also contains 0.1% (v/v) Tween 20 (sorbitan

monolaurate polyoxyalkylene). Within a response time of 30 seconds, a fluorescence peak was observed, the height of which was found to correspond to the enzyme activity.

CLAIMS

1. A method for monitoring for the presence of an enzyme, comprising producing a substrate for the enzyme, which substrate has been modified by labelling it with a fluorophore, exposing the labelled substrate to the enzyme, and monitoring the fluorescent-labelled reaction products, thereby produced.

2. A method as claimed in Claim 1, wherein the fluorescent-labelled reaction products are monitored by means of a fluorimeter.

3. A method as claimed in either of the foregoing claims, wherein the enzyme is a protease, cellulase, lipase, collagenase or amylase enzyme.

4. A method as claimed in Claim 3, wherein the enzyme is a protease enzyme and the substrate is a protein.

5. A method as claimed in Claim 4, wherein the protease enzyme is subtilisin, esperase, trypsin or alcalase.

6. A method as claimed in Claim 4 or Claim 5, wherein the protein is gelatin, porcine thyroglobulin or bovine serum albumin.

7. A method as claimed in Claim 3, wherein the enzyme is a cellulase enzyme and the substrate is cellulose.

8. A method as claimed in Claim 3, wherein the enzyme is a collagenase enzyme and the substrate is collagen.

9. A method as claimed in any of the preceding claims, wherein the fluorophore is lucifer yellow.

10. A method as claimed in any of Claims 1 to 8, wherein the fluorophore is fluorescein or a fluorescein derivative.

11. A method as claimed in any of the preceding claims, carried out on a continuous basis by conveying air containing the enzyme continuously into a solution and then passing the resulting enzyme solution over a fixed bed of the labelled substrate.

12. A method as claimed in any of the preceding claims, wherein the labelled substrate is carried on a support.

13. A method as claimed in Claim 12, wherein the support comprises glass beads or comprises cellulose particles containing iron oxide.

14. A method as claimed in Claim 12, wherein the support is an optical fibre.

15. A method as claimed in any of Claims 11 to 14, wherein the solution contains a buffer.

16. A method as claimed in Claim 15, wherein the buffer is an acetate buffer, a phosphate-buffered saline or tris-(hydroxymethyl)-aminomethane.

17. A method for monitoring for the presence of an enzyme, which method is substantially as hereinbefore described in any of the Examples.

The Patent Office

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Application No: GB 9625682.1 **Examiner:** Colin Sherrington
Claims searched: 1 to 17 **Date of search:** 4 March 1997

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): G1B(BAB,BAG,BAH)

Int Cl (Ed.6): C12Q 1/34,1/37,1/40

Other: ONLINE: WPI,CLAIMS

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
X	GB 2133007 A	(BAYER AKTIENGESELLSCHAFT) -whole document	1 (at least)
X	GB 2166545 A	(GENZYME CORPORATION) -whole document	1 (at least)
X	EP 0037583 A1	(KINNUNEN, PAAVO K.J. *et al.*) -whole document	1 (at least)
X	EP 0596355 A1	(GENENTECH, INC.) -whole document	1 (at least)
X	WO 80/00351 A1	(AMERICAN HOSPITAL SUPPLY CORPORATION) -whole document	1 (at least)
X	WO 80/02295 A1	(SMITH, ROBERT E.) -whole document	1 (at least)
X	WO 91/16336 A1	(CARLSBERG A/S) -whole document	1 (at least)
P,X	WO 96/13607 A1	(ONCOIMMUNIN, INC.) -whole document	1 (at least)
X	US 5164300	(WASHINGTON UNIVERSITY) -whole document	1 (at least)
X	US 5236827	(BECTON, DICKINSON AND COMPANY) -whole document	1, 11 (at least)

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

The Patent Office

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Application No: GB 9625682.1 **Examiner:** Colin Sherrington
Claims searched: 1 to 17 **Date of search:** 4 March 1997

Category	Identity of document and relevant passage		Relevant to claims
X	US 5399487	(HAEMATOLOGIC TECHNOLOGIES, INC.) - whole document	1 (atleast)

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.